Drugs Against Cancer: Stories of Discovery and the Quest for a Cure

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CHAPTER 10

The Topoisomerase II Story: from methodology to a new anticancer drug target.

Introduction

The DNA double helix is naturally twisted. Normally, it has one full twist for every 10.5 base-pairs. But what happens to the twists when the DNA strands are pulled apart during replication or transcription? It is like trying to pull apart a long 2-strand twisted rope. The twists could bunch up becoming harder and harder to pull the two strands of the rope apart.

Soon after Watson and Crick solved the structure of double-stranded DNA, Max Delbruck pointed out that the number of twists in the parental DNA helix would still be present after the DNA duplicated (Delbruck, 1954). Those twists would somehow have to be eliminated to allow the pair of newly replicated DNA helices to separate. Delbruck concluded that, in order to accomplish that trick, the cell must have a way to transiently cut DNA strands and allow strands to pass through the gap. Many years later, enzymes that accomplish that feat were discovered and came to be called topoisomerases.

Two general types of topoisomerases were discovered. Type I topoisomerases transiently cut one strand of double-stranded DNA helix and allow the other strand to pass through the gap. Those topoisomerases of type I are the topic of the next chapter. In the current chapter, I focus on type II topoisomerases that transiently cut both strands of a double-stranded DNA helix and allow another double-stranded DNA helix to pass through the gap. That was the type of topoisomerase activity we

discovered to be targeted by several anticancer drugs. I will tell how we came to that finding in the first part of this chapter.

It was hard to imagine how enzymes could possibly break the DNA while keeping hold of both ends of the broken DNA to prevent them from drifting away from each other, because the ends had to be rapidly resealed after the strand passage. Nature, however, as usual, found a straight-forward solution.

When the DNA has replicated and the chromosomes begin to condense on their way to mitosis, the mother and daughter strands remain entangled in a manner that pulling the long DNA stands apart becomes like trying to pull two interlocked rings apart. It can't be done without cutting one of the rings. Type II topoisomerases manage to cut both strands of a DNA double-helix, allow another DNA double-helix to pass through the gap, and then quickly reseal the broken ends.

An extreme case where a type II topoisomerase is required is the remarkable DNA structure in the mitochondrium of trypanosomes, one species of which causes sleeping sickness, transmitted through the bite of the tsetse fly. The structure, called a kinetoplast, consists of a large number of interlocked circles. When the organism divides, its kinetoplast also duplicates. But for that to happen, the grossly interlocked DNA circles have to be disentangled by transiently cutting DNA helices, which is what a type II topoisomerase does (Figure 10.1).

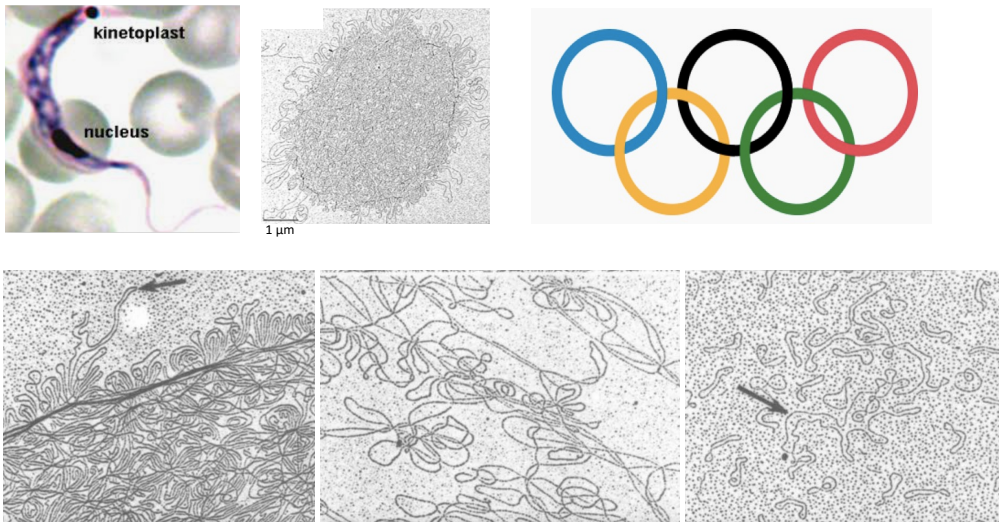

Figure 10.1. An extreme case where a type II topoisomerase is urgently needed. The kinetoplast of a trypanosome is its mitochondrial DNA, which consists of a large number of interlocked (catenated) DNA circles. When the kinetoplast duplicates during the organism's division, a type II topoisomerase transiently cuts those DNA circles to allow them to disentangle.
Upper panels: *left*, a trypanosome among red blood cells (which are 7 µm in diameter); *center*, what a kinetoplast looks like when spread out in an electron microscope; *right*, the

ring symbol of the Olympics (jeux olympiques), illustrating how the kinetoplast DNA circles are interlocked.
Lower panels: *left*, edge of an untreated kinetoplast; *center*, brief treatment with a type II topoisomerase; *right*, full treatment. (From (Marini et al., 1980))

Interest in topoisomerases blossomed when we discovered that some important anticancer drugs work by blocking one or another of those two types of topoisomerases. This chapter is about drugs that block topoisomerase II, which was the first topoisomerase found to be blocked by some anticancer drugs. The following chapter (Chapter 11) will be about drugs that bock type I topoisomerases.

Discovery

The topoisomerase enzymes themselves were discovered before there was any indication that they might be targets of drug action. Topoisomerase type I enzymes were first to be discovered. They were discovered in bacteria and viruses and were initially called "DNA nicking-closing enzymes" or “DNA swivelases” (Champoux, 1978b; Champoux and Dulbecco, 1972; Radding, 1978). As explained by Champoux, the enzymes "introduce a transient single-strand break in duplex DNA and thereby provide a swivel for helix unwinding (DNA swivelase)" (Champoux, 1978b). Those names were later replaced by "topoisomerase" to indicate that the enzyme changes the topology of the DNA (a change in topology of an object occurs when the object has to be cut to make the change).

Type II topoisomerases, discovered later, cleave both strands of the DNA so as to form a double-strand break through which another double-stranded DNA can pass before the enzyme reseals the break (Liu et al., 1980; Miller et al., 1981). This amazing ability is important during and after DNA replication, because the new chromosomal DNA would otherwise remain entangled in loops analogous to the interlocking circles in the symbol of the Olympics (Figure 10.1), the interlocking would hinder the proper separation of chromosomes during mitosis. Although the nuclear DNA molecules of animal cells are not circular, they are so very long that, when duplicated, they can only be disentangled by transient cutting and strand passage. How topoisomerase II accomplishes that trick will be explained later in this chapter.

First clues of anticancer drugs acting on topoisomerases.

The previous chapter explained how we discovered that DNA strand breaks increase the rate at which the DNA from lysed cells can pass through filter pores, and how we used this phenomenon to work out methods to quantify the frequencies of both DNA single-strand breaks and DNA-protein crosslinks produced by various DNA-damaging drugs and agents. Quite unexpectedly, those studies led us to discover

drug actions that we attributed to an effect on an enzyme that had the properties of a nicking-closing enzyme (later called topoisomerase); such enzymes were known in bacteria but had not yet been found in animal cells. Here is how that discovery came about:

In 1978, a young physician, Warren E. Ross, having completed his first year as a Clinical Associate in the National Cancer Institute, joined my laboratory to gain some research experience. At that time, we were studying DNA damage and repair produced by various anticancer drugs in cells. We had developed a new technique using filters that allowed us to measure DNA stand breaks and DNA crosslinks, both between the paired strands, and between DNA and proteins (Kohn and Ewig, 1979). The story of that technique was told in the previous chapter (Chapter 9).

Warren wanted to apply that methodology to doxorubicin, a promising drug that interested him in his Clinical Associate year. Doxorubicin had been reported to break DNA strands in studies that used a previous less precise and less sensitive ultracentrifugation method. We fully expected that using our new filter-based technique, we would easily confirm the production of DNA breaks by doxorubicin in mammalian cells, as had invariably been the case with several other DNA-breaking agents that we had tested (Erickson et al., 1977; Fornace et al., 1976). However, Warren's repeated attempts to confirm doxorubicin-induced DNA breaks using our filter method failed to show any sign of DNA breakage whatsoever (arrow in the *left* panel of Figure 10.2).

His experiment however suggested that doxorubicin produced DNA-protein crosslinks: the lower two curves in the *left* panel of Figure 10.2, showed that using x-rays to produce strand breaks yielded less than the expected rate of elution (see legend to Figure 10.2) . We thought that doxorubicin failed to show any DNA strand breaks, because the drug might have produced an excess of DNA-protein crosslinks, which could have hidden the strand breaks -- because the DNA-bound proteins could have caused all of the DNA fragments to stick to the filter.

That idea seemed to be confirmed, because digesting the lysed cells with a proteinase before alkaline elution, produced an increased elution rate that confirmed the production of DNA strand breaks by doxorubicin (*right* panel of Figure 10.2). Moreover, when Warren applied our protocol for protein-digestion (see Chapter 9), the results were astounding: doxorubicin then produced a beautiful pattern of dose-dependent strand breakage (Figure 10.3). But protein digestion was needed to reveal those breaks – because the DNA fragments were completely hidden by being linked to proteins that stuck to the filter.

In order to hide the strand breaks so completely, however, we thought a large excess of DNA-protein crosslinks relative to strand breaks would be needed. We were able to check on that, because we had recently worked out how to quantify both strand breaks and DNA-protein crosslinks (Chapter 9) (Kohn and Ewig, 1979).

Our ability to quantify the strand breaks and DNA-protein crosslinks became essential to attributing the drug action to a nicking-closing enzyme (topoisomerase).

The results of those quantifications at first presented a big surprise and a puzzle. They showed that there was NO excess of DNA-protein crosslinks over strand breaks. In fact, repeated measurements with doxorubicin, as well as some other DNA intercalators (such as ellipticine) consistently showed that the number of the two types of DNA lesions were equal, within experimental error!

I thought that amazing and suspected that there must be some connection between the strand breaks and the DNA-protein crosslinks. They must be causally connected in some way.

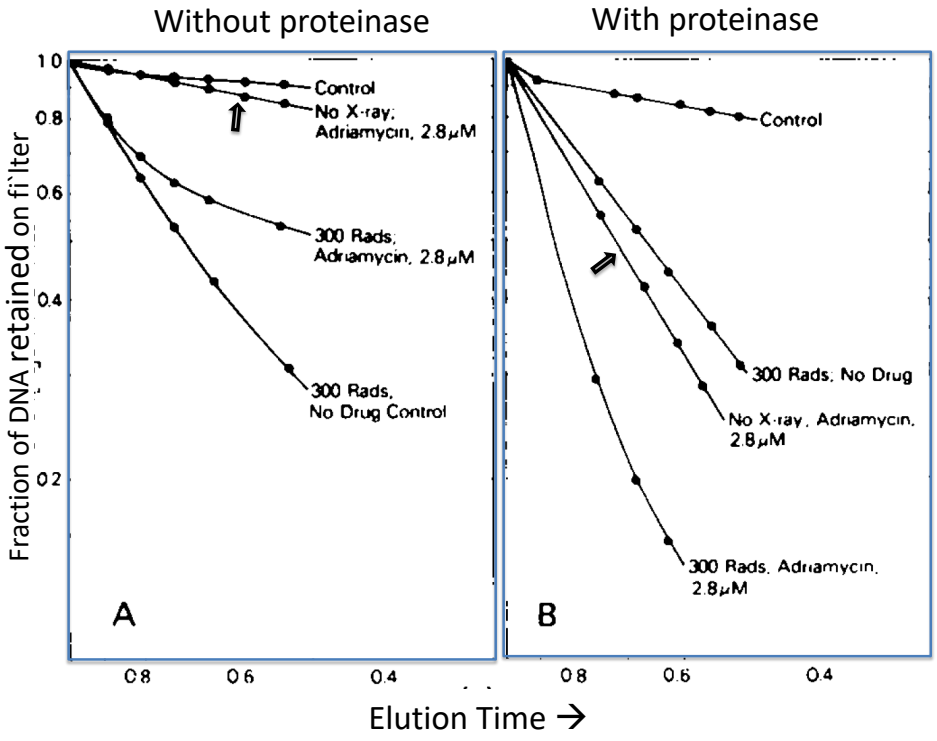

Figure 10.2. This experiment by Warren E. Ross in 1977 in my laboratory showed, surprisingly, that doxorubicin (Adriamycin) at first showed no increase in DNA alkaline elution rate, thus no indication of any strand breaks (arrow in *left* panel). However, the elution rate of the DNA after subjecting the cells to 300 rad of x-rays just before lysis of the cells was reduced in the doxorubicin-treated cells, which suggested the presence of DNA-protein crosslinks (lower two curved in the *left* panel). When the assays included digestion of the lysed cells with proteinase, however, doxorubicin showed the increased DNA elution rate expected for the presence of DNA stand breaks (arrow in the *right* panel). All together, these results suggested that doxorubicin produced both strand breaks and DNA-protein crosslinks (Ross et al., 1978).

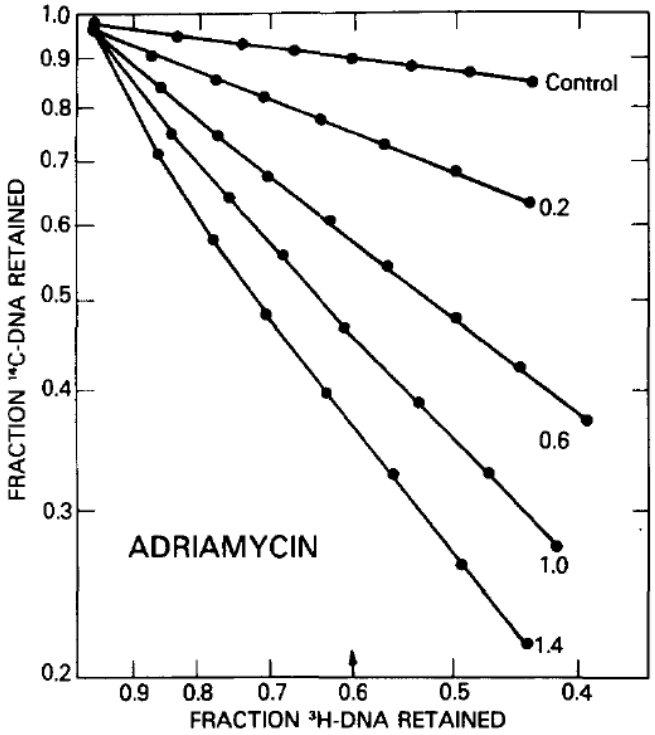

Figure 10.3. Doxorubicin-induced DNA strand breaks were beautifully revealed after the DNA-linked protein was digested away. In this experiment, doxorubicin-treated cells that were then lysed on the filter were subjected to a protein-digesting enzyme (proteinase K) before pumping an alkaline solution through the filter (as described in Chapter 9). DNA strand breaks are seen to be in proportion to the doxorubicin dose. When the protein-digestion step was omitted, no DNA strand breaks could be seen (elution curve similar to that labeled "control") (Ross et al., 1979; Ross et al., 1978). (Adriamycin is another name for doxorubicin.)

The next notion that dawned was that maybe the DNA-linked protein was actually an enzyme that produced the strand break and that the drug caused the enzyme to remain linked to one end of the break it produced. Then every DNA strand segment would have a protein linked to it and the number breaks and DNA-protein crosslinks would be equal, as observed in our experiments (Ross et al., 1979).

It is not often that one experiences the delight of imagining something important that perhaps no one had thought of before and having it come to fruition.

But to verify that idea required some calculation. Three models could be considered for the distribution of the strand breaks and the DNA-protein crosslinks (Ross et al., 1979). The models are described in Figure 10.4. Model I assumed a random distribution of both strand breaks and DNA-protein crosslinks; this model failed,

because an equal frequency of the two DNA lesions would have left some DNA strands without protein links, contrary to our evidence. Model III assumed one protein bound to every strand segment anywhere along the segment; this seemed an unlikely circumstance, because it was difficult see what could have brought about such an arrangement. Model II was plausible if the linked protein was in fact an enzyme that produced the breaks and if doxorubicin caused the enzyme to remain attached to one end of the break it produced.

To recapitulate, the equal frequency we observed of doxorubicin-induced strand breaks and DNA-protein crosslinks was at first puzzling, because if the two types of DNA lesions were randomly distributed along the DNA, some broken strands would by chance have been free of protein and therefore should have passed though the filter, contrary to our observations. I then reasoned that the breaks could have been completely hidden, as observed, if there were just one protein linked to each broken strand. That, at first seemed unlikely, but I soon realized that it could be the case if each protein molecule were bound consistently to one end of each break site (Figure 10.4, Model II). Algebraic analysis of our data was consistent with that possibility (Ross et al., 1979; Ross et al., 1978). Support for that idea came from measurements of several other drugs that too produced DNA strand breaks that were hidden by DNA-linked protein with equal frequencies of DNA strand breaks and DNA-protein crosslinks.

As already said, if a protein were linked consistently to one end (5’ or 3’) of each strand break, then perhaps the protein was an enzyme that produced the break. An enzyme with that property had already been described time in bacteria: the already mentioned “DNA nicking-closing enzyme” or “DNA swivelase” (Champoux and Dulbecco, 1972).

We therefore proposed that a type of nicking-closing enzyme existed in mammalian cells and that doxorubicin (as well as other DNA intercalating agents that we observed to produce similar results (equal numbers of DNA strand breaks and DNA-protein crosslinks) caused the enzyme to become blocked in an intermediate state where the break had been produced but had not yet resealed. Therefore in 1979 we "proposed that intercalation-induced distortion of the DNA helix leads to strand scission by a nuclease which becomes bound to one terminus of the break so as to form a DNA-protein crosslink" (Ross et al., 1979). Nicking-closing enzymes (also called "swivelases" or "DNA unwinding enzymes"), were soon found in mammalian cells (Champoux, 1978a) and were later dubbed “topoisomerases.”

Those studies gave the first clue that drugs, such as doxorubicin, trap a topoisomerase in a state where the DNA strands are cleaved while the enzyme remains bound to the ends of the broken strands. (The discovery of topoisomerases would not by itself have suggested that those enzymes might be therapeutic targets of certain anticancer drugs.)

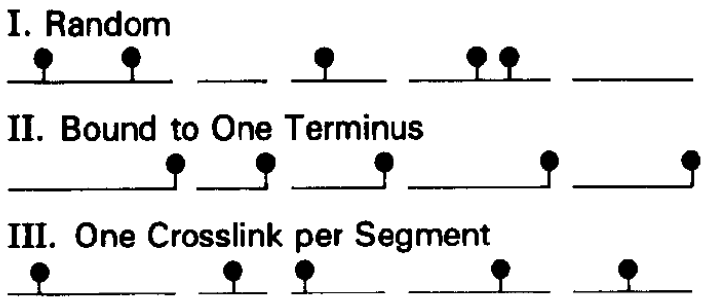

Figure 10.4. I considered three models to account for our observation that doxorubicin (as well as some other DNA intercalators, such as ellipticine) produced equal numbers of strand breaks and DNA-protein crosslinks. The lines in the diagrams represent DNA strands with interruptions at break sites. The black circles represent protein molecules bound to the DNA strands. Model I was for proteins bound at random places on the DNA; note that by chance some broken DNA pieces have no protein attached. Model III for one and only one protein randomly placed on each DNA segment was unlikely, because how would the linked protein know where the breaks were located? Model II, the bound-to-one-terminus model, was for a protein bound consistently to one end of each break. Quantitative examination of the data together with some algebra was consistent only with Model II. The conclusion that there was a protein bound consistently to one end of each break (Model II) suggested that the DNA-bound protein molecules were in fact enzyme molecules that produced the breaks and remained bound consistently to one end (5' or 3') (Ross et al., 1979), as was later found to be the case for topoisomerases.

The next step was to demonstrate the effect of the drugs on purified topoisomerase enzyme or in solutions extracted from cells containing the enzyme. Janek Filipski, a Polish visiting scientist in our lab experienced considerable frustration trying to accomplish this. He succeeded in showing that cell extracts contained an enzyme that produced the expected drug effects – DNA stand breaks with associated DNA-protein crosslinks. However, when he tested the then-known topoisomerase enzyme, the drugs had no effect (Filipski et al., 1983a, b).

Soon after he published that work, however, the difficulty was revealed: there were in fact two kinds of topoisomerases, and the one he tested – the only one that was known at the time -- was the wrong one. Only topoisomerase I was known at the time of his experiments. But the enzyme the drugs acted on was topoisomerase II exclusively. That new enzyme was being discovered, unbeknownst to us, during the latter part of his studies.

In 1980, Leroy Liu, working with Bruce Alberts at the University of California in San Francisco, had isolated the enzyme that came to be known as topoisomerase II (Liu et al., 1980). About 2 years later, after Leroy Liu had moved to Johns Hopkins University in Baltimore, I visited his laboratory and we discussed the possibility that the drug effects that we could not attribute to actions on topoisomerase I were

actually caused by actions on his topoisomerase II. After preliminary experiments to get the drug treatment conditions right, Leroy Liu and his colleagues, as well as John Minford, Yves Pommier and Leonard Zwelling in my laboratory, soon confirmed that indeed doxorubicin trapped topoisomerase II bound to one end of a DNA break, an intermediate state in the enzyme's breakage/resealing cycle (Minford et al., 1986; Nelson et al., 1984; Tewey et al., 1984a; Tewey et al., 1984b). In addition to doxorubicin, we found that some other DNA intercalating drugs, such as amsacrine (m-AMSA) and ellipticine, also trapped topoisomerase II DNA-cleavage complexes in a fashion similar to doxorubicin (Pommier et al., 1985).

The nature of the protein-linked DNA strand breaks that we attributed to trapping to topoisomerase II was further revealed by studies of the action of amsacrine (m-AMSA) by Leonard Zwelling in my laboratory (Zwelling et al., 1981). Len added m-AMSA to cultures of mouse leukemia cells and measured the production of protein-linked DNA strand breaks using our filter methods (Kohn, 1996) (Figure 10.5). If m-AMSA produced DNA breaks like an ordinary DNA damaging agent, the breaks would continue to accumulate while active agent was present. He found, however, that the breaks produced by m-AMSA soon leveled off, and then remained at a constant level as long as the drug was present. When the drug was removed, the breaks rapidly vanished, showing that the enzyme continued to be active and was necessary to maintain the high plateau levels of protein-linked DNA strand breaks. We concluded that, in the presence of m-AMSA, there was a rapid equilibrium between the formation and the reversal of the DNA breaks. The simplest explanation was that the drug bound to and trapped an intermediate state of an enzyme that continually opened and closed DNA breaks. In other words, the drug stabilized a state of the enzyme between cleavage and resealing of the DNA. Ordinarily, the enzyme resealed the breaks so quickly that one did not see the cleaved state. With drug bound to the enzyme, the duration of the cleaved state was prolonged, producing the steady-states seen in Figure 10.5.

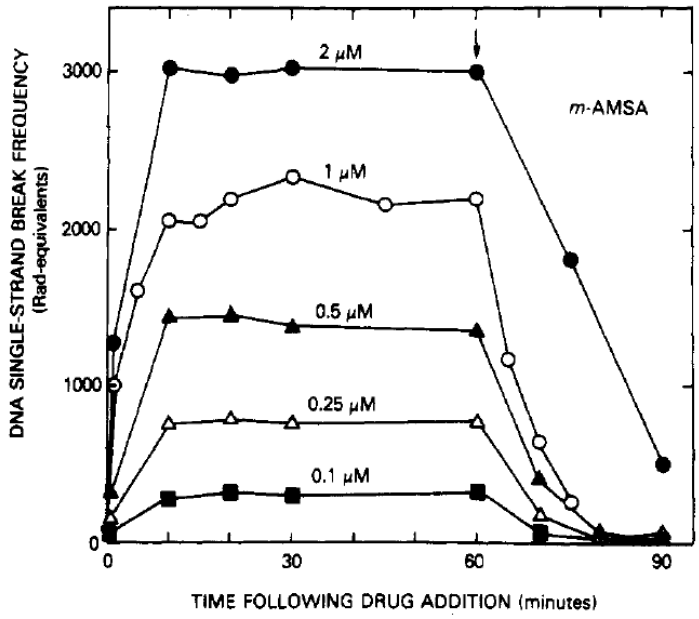

Figure 10.5 Treatment of cells with m-AMSA (amsacrine) caused DNA strand breaks to appear and reseal rapidly, consistent with an effect on a topoisomerase. In the continued presence of drugs, the number of strand breaks increased and soon reached a plateau that was higher when the drug concentration was higher. After 60 minutes, when the drug was removed (arrow), the strand breaks soon vanished. This result showed that there was a rapid equilibrium between formation and reversal of the strand breaks, and the number of strand breaks at equilibrium increased with drug concentration (Zwelling et al., 1981). An ordinary DNA damaging agent would have continued to increase the number of strand breaks, in contrast to the flat equilibria seen here.

Later it turned out that another drug, camptothecin, trapped topoisomerase I in a reversible reaction where only one of the strands of the DNA double helix was cleaved. Topoisomerase I, like topoisomerase II, undid excessive DNA twists, but did so by producing DNA single-strand breaks, as opposed to the double-strand breaks produced topoisomerase II. The camptothecin story is related in the next chapter.

How doxorubicin and other intercalator-type drugs trap DNA-topoisomerase II complexes.

In 1989, when purified topoisomerase II and DNA sequencing gels had become available, we wondered whether the drugs had preferences for the DNA sequences where they incited the enzyme to cleave the DNA. We found that DNA cleavage sites did indeed occur at preferential sites (Figure 10.5).

To determine whether the enzyme preferred to cleave at particular DNA sequences , we examined a large number of topoisomerase II DNA cleavage sites trapped by

various intercalator-type drugs (Capranico et al., 1990a; Capranico et al., 1990b) (Pommier et al., 1991). Figure 10.6 shows one of our first DNA sequencing gels that indicated exactly where in the DNA sequence the drug-induced cleavage sites were located. When we began that investigation, however, we did not suspect that it was to give a clue to the structure of the trapped DNA-topoisomerase complex itself.

Our first notable observation was that doxorubicin breaks occurred preferentially where there was an A (adenine) adjacent to the cleavage site on the side toward the 5' end of the DNA strand. For amsacrine (m-AMSA) there was also a preference for a particular base at the cleavage site, but in that case the preference was for an A on the side towards the 3' end of the broken DNA strand. For etoposide and teniposide (VP-16 and VM-26), again there was a preference for a particular base at the cleavage site, but the preference in that case was for a C on the 5' side (Figure 10.7). Those findings were exciting, because they had the feel of a mathematical quality, like a code of some kind.

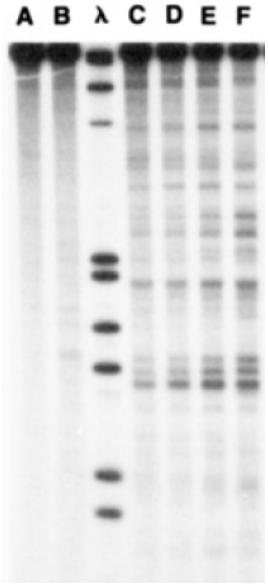

Figure 10.6. One of our first electrophoretic DNA sequencing gels showing cleavage of DNA at specific sites induced by mammalian topoisomerase II in the presence of doxorubicin (Capranico et al., 1990b). **A**, DNA alone. **B**, DNA plus topoisomerase II; these 2 lanes show that neither DNA alone nor topoisomerase alone nor DNA with only drug, caused breaks. **C-F**, DNA plus topoisomerase II plus increasing concentrations of doxorubicin; the bands show where in the DNA sequence cleavage occurred in the presence of topoisomerase II plus doxorubicin. As the concentration of doxorubicin was increased, the bands became darker, indicating increased frequency of breaks at those sites. (The lane labeled λ shows marker bands for determination of the exact positions of the cleavage sites in the DNA sequence.)

The preference for a base on one side or the other of the break site, and its dependence on the identity of the drug, suggested that the drug molecule stacks against one side or the other of the break site the way DNA intercalators stack against the base-pairs (Pommier et al., 1991; Pommier et al., 2000). We guessed (correctly) that the drug stacked against a particular base-pair at the cleavage site (Figure 10.7), as later shown in a crystallographic structure .

The drug plastered against a base-pair at the end of the break prevented the topoisomerase II from closing the break. The drug thus trapped the DNA-topoisomerase complex in a state where the DNA was cleaved and could not reseal. Since the bindings were reversible, the drug eventually dissociated and allowed the break to reseal, as proved in Figure 10.5.

The cell however would not rely on the spontaneous dissociation of the drug, because it took some time, during which an encounter with a transcription or replication fork could have lethal consequences, as will be explained later in this chapter. The cell therefore has repair machinery to clean up (albeit slowly) the trapped complexes.

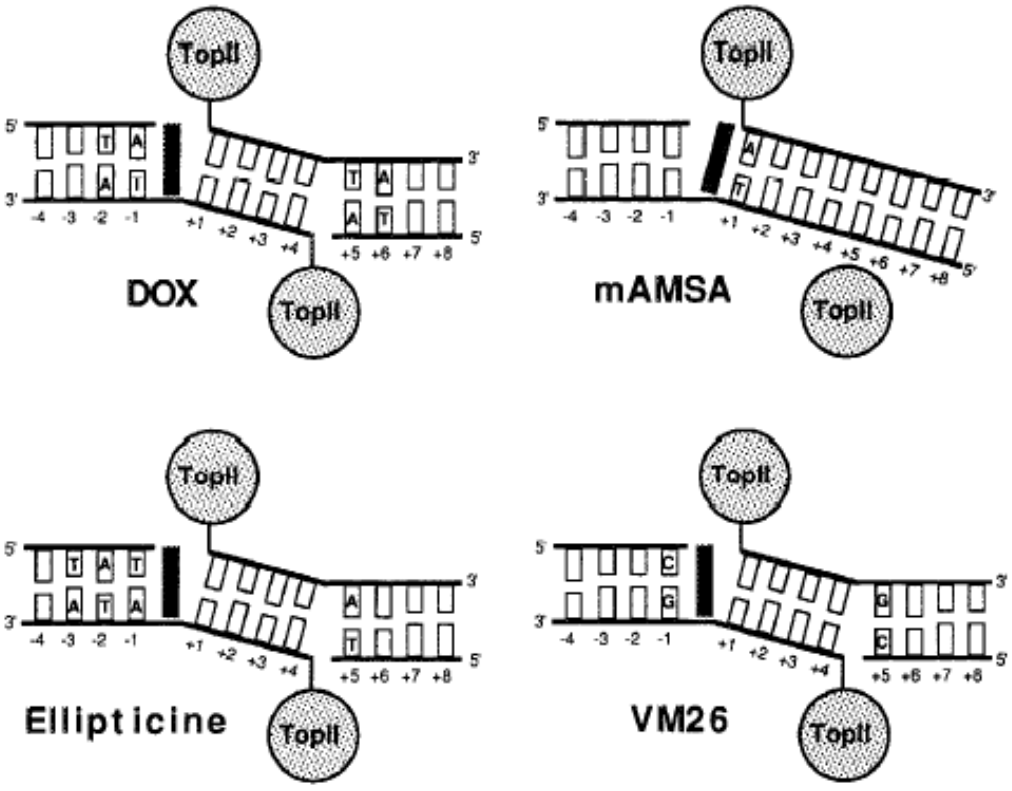

Figure 10.7. Preferred positions of the drugs (solid rectangles) at the drug-topoisomerase II cleavage sites. We inferred these configurations from our observed site preference observations (such as shown in Figure 10.5 (Pommier et al., 1991)). This model was later confirmed by x-ray crystallography (Wu et al., 2013). The DNA base preferences for the immediate neighbors at the break site were, as indicated in the figure: for doxorubicin (DOX), A on the 5' side of the break; for amsacrine (m-AMSA), A on the 3' side; for ellipticine, T on the 5' side; for teniposide (VM26) and etoposide (VP16), C on the 3' side. Topoisomerase II consists of two identical molecules bound together but here shown separated for clarity (Figure 10.7): one cleaves one DNA strand, and the other cleaves the other strand. The two cleavage sites were always separated by 4 base-pairs, and the base preferences were similar at the two sites.

How type-2 topoisomerases undo entangled DNA helices.

The problem of separating interlocked newly replicated DNA loops (Figure 10.1) at first seemed almost insurmountable, but topoisomerase II manages to do it! It is like a conjuring trick that passes one rope through the middle of another. How one DNA double strand could be made to pass through another, while keeping hold of the strand ends, was at first hard to imagine. But, as so often is the case, evolution discovered a solution, which turned out to be quite simple.

It was discovered that the magic happens through the cooperation of two identical topoisomerase II molecules (Figure 10.8): the topoisomerase molecules first cut one DNA double-helix (green), then allow the other (red) to pass through the gap and out the other side; then the molecules quickly and perfectly make the green DNA whole again. It happens quickly and perfectly. The key is that two topoisomerase molecules cooperate so that the cut DNA ends are always bound to the topoisomerases and never free to drift apart, and that the topoisomerase II pair of molecules have two places where they can bind each other alternately to let the passing double helix come in from one side and out the other (Figure 10.8).

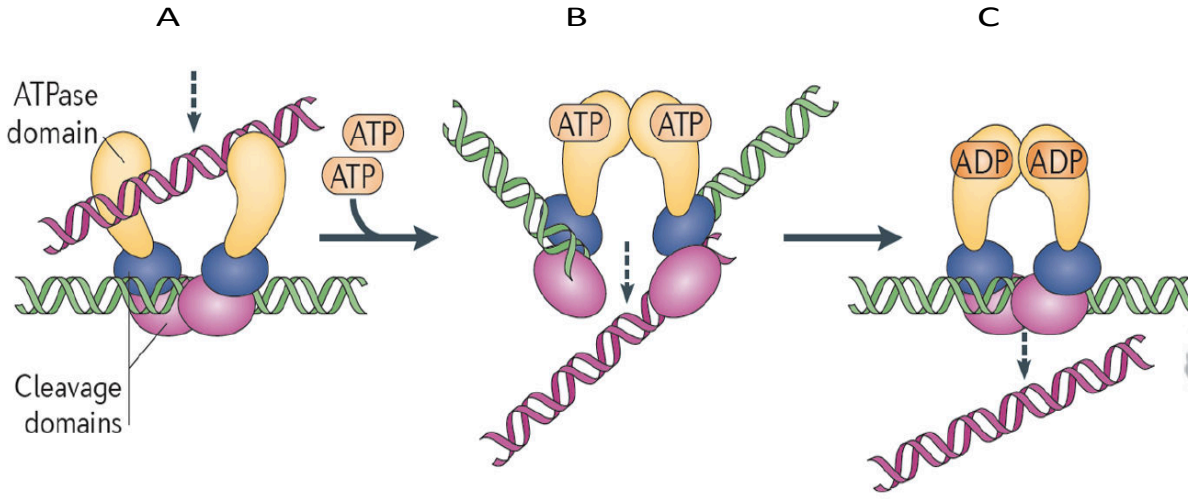

Figure 10.8. How topoisomerase II passes one DNA double helix (red) through another (green) (Vos et al., 2011). Two identical topoisomerase molecules cooperate to accomplish this magic. The ATP/ADP units provide the energy that drives the machine. (*From Nature Reviews Molec Cell Biol 2011.*)

Doxorubicin and other DNA intercalation-type drugs bind to an intermediate state (such as B, in Figure 10.8), where the DNA is broken; the bound drug prevents the break from being resealed. Figure 10.8. shows the structure of this intermediate state as revealed by x-ray crystallography; we were happy to see our inferred model (Figure 10.7) confirmed by x-ray crystallography (Figure 10.9).

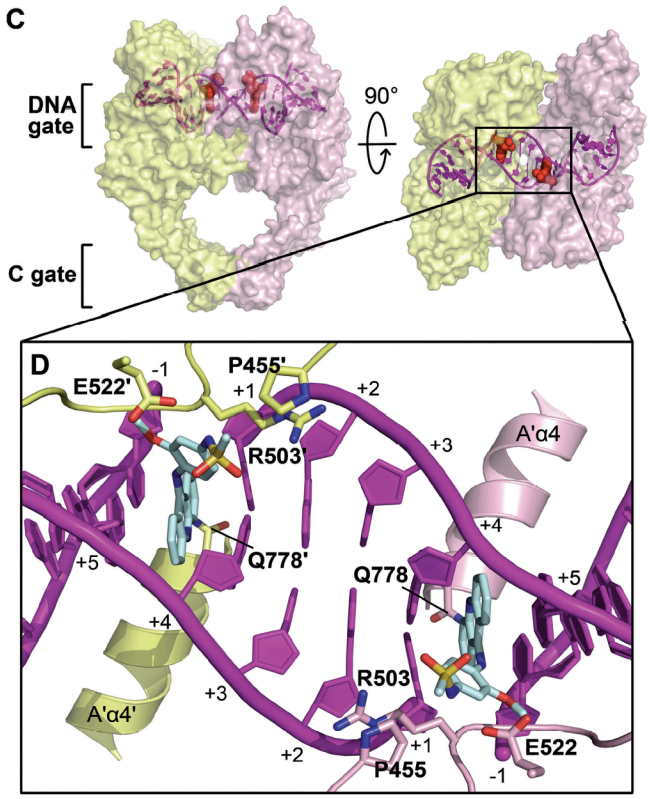

Figure 10.9. Structure of DNA-topoisomerase II (Top2) trapped by amsacrine (m-AMSA) in a state where both DNA strands are cleaved (Wu et al., 2013). The structure was based on x-ray crystallography. The upper part of the figure shows the topoisomerase II homodimer (yellow and pink) and the bound DNA (red). Below is a detailed view of the cleaved DNA with amacrine intercalated as we had surmised in Figure 10.7. The DNA (maroon) is shown with the base-pairs edge-on connected to the DNA backbone via the pentagonal deoxyribose units. Two amsacrine molecules (blue) are DNA-bound at the two break sites, which are separated by 4 base-pairs as we saw in Figure 10.7. In the absence of drug, those 4 base-pairs would come apart and the DNA double-strand break would open and allow another DNA helix to pass through. The complementarity of those 4 base-pairs then helps the two parts of the broken strand to fit together and restore the original unbroken DNA. Two alpha-helical parts of the topoisomerase II protein that interact with the DNA and/or amsacrine at the break sites are shown in yellow and pink (Wu et al., 2013).

How the topoisomerases, their structure and functions were discovered.

The story goes back to 1969, when James C. Wang, then at the University of California at Berkeley discovered an enzyme activity in E. coli extracts that relaxed over-twisted (supercoiled) DNA. Two years later, he had purified the enzyme and called it omega protein (Wang, 1969, 1971). He knew that to relax this circular

supercoiled double-stranded DNA, a strand had to be cut to allow the DNA to relieve its excessive twists and relax to its normal degree of twisting (about 10.5 base-pairs per twist). He thought at first that two enzymes were needed: one to cut a strand and another to re-ligate the broken strand after the DNA spontaneously relieved its excessive twists, but soon found that it was all done by his single purified enzyme. Moreover, the DNA-relaxing activity of the enzyme did not require energy – which a ligase enzyme would have required.

His new purified enzyme needed a name. His name for the enzyme – omega protein – had a non-committal quality to it, and so, that name was soon replaced by names reflecting what the enzyme did: DNA-unwinding protein or DNA swivelase. After more investigation by several researchers, its name settled down to the modern: topoisomerase I.

Topoisomerase 1 relaxed supercoiled double-helical DNA by passing one of the strands through a gap created by cleaving the other strand of the same double-helix. In 1980, as already mentioned, Leroy Liu and Bruce Alberts, discovered another type of topoisomerase, which functioned by passing one double-helix through a break created in both strands of another double-helix. They called their new enzyme topoisomerase II. In 1983, Leroy Liu, having moved to Johns Hopkins University in Baltimore, Maryland, had led his research group to carry out the purification of topoisomerase II from several types of mammalian cells (Liu et al., 1983). The new topoisomerase served to disentangle DNA during entry into mitosis (Champoux, 2001) and, as described above, was the enzyme that his laboratory and mine discovered to be targets for anticancer drugs, such as doxorubicin.

How drugs that poison topoisomerases kill cancer cells.

You might think that the toxic effects of a drug that poisons an enzyme would be overcome if the cell increased the amount of the enzyme, so that some enzyme activity would still be retained even in the presence to the drug. According to that viewpoint, cells would become resistant to the drug if the amount of the drug's target enzyme were increased, which is often the case of other enzymes. However, for topoisomerases the opposite was found to be true. Cells became drug-resistant if they *reduced* the amount of topoisomerase they made, because it was the drug-topoisomerase combination that was toxic to the cell (Nitiss, 2009) (Pommier, 2013).

But why would a drug-topoisomerase complex, sitting quietly on the DNA cause trouble? The trouble arose when a DNA replication or transcription machine came along and encountered one of those complexes. The encounter created an abnormal DNA structure, such as a double-strand end, which was hard to repair, and such lesions in the DNA could ultimately kill the cell (Hsiang et al., 1989).

How cells defend against drugs that poison topoisomerases.

Three processes were discovered that helped prevent poisoned topoisomerase from leading to a lethal outcome. First, the cell had enzymes that removed the trapped topoisomerase from the DNA before anything bad happened. Second, if a DNA replication or transcription machine had already collided with a drug-trapped topoisomerase, a DNA repair mechanism -- DNA nucleotide excision repair – was found to come into play to restore the integrity of the DNA. Third, a defense against a lethal outcome was initiated by signals to the cell cycle control systems to delay replication and mitosis, so as to give more time for repair to take place before disastrous consequences occur. If there were too many trapped complexes to handle, however, the cell could give up and undergo programmed suicide (apoptosis).

The first countermeasure mentioned above -- removal of the trapped topoisomerase – became fairly well understood. It was found to be accomplished by enzymes called tyrosine-DNA-phosphodiesterases (TDP1 and TDP2). Phosphodiester bonds normally link between nucleotide units in the DNA sequence When a DNA-topoisomerase complex has cleaved a DNA strand, a phosphodiester bond links one end of the cleaved DNA to a tyrosine amino acid of the topoisomerase. TDP1 and TDP2 juggled the phosphodiester bond to make the topoisomerase protein come off (at which point the drug also came off), which then allowed the DNA break to reseal. When the DNA strand break could not close because of an intercalated drug, TDP1 or TDP2 would break the bond between the DNA end and the topoisomerase's tyrosine. The importance of this action was shown in a report that TDP2 helps cells survive topoisomerase II trapping by the Top2 blocker, etoposide (Kont et al., 2016) (Figure 10.10).

Actually, the process was a bit more complicated. Before the TDP1 or TDP2 could have access to cleave the tyrosine bond to the DNA, a large part of the topoisomerase protein had to be digested away. This was done by an important (and amazing) machine in the cell, called a proteasome.

(Other types of DNA damage, such as produced by alkylating agents can also trap topoisomerases (Schellenberg et al., 2016), but that is generally a minor action relative to other effects of those agents.)

The second defense: nucleotide excision repair, as well as the third defense: signaling to the cell cycle control system to delay replication and the initiation of apoptosis will be the subjects of later chapters. Much effort aimed to unravel the complexities of how the DNA lesions caused by topoisomerase-trapping drugs signaled to the DNA repair and cell cycle control systems to initiate further survival actions in the cell (Cristini et al., 2016) (Sakasai and Iwabuchi, 2016).

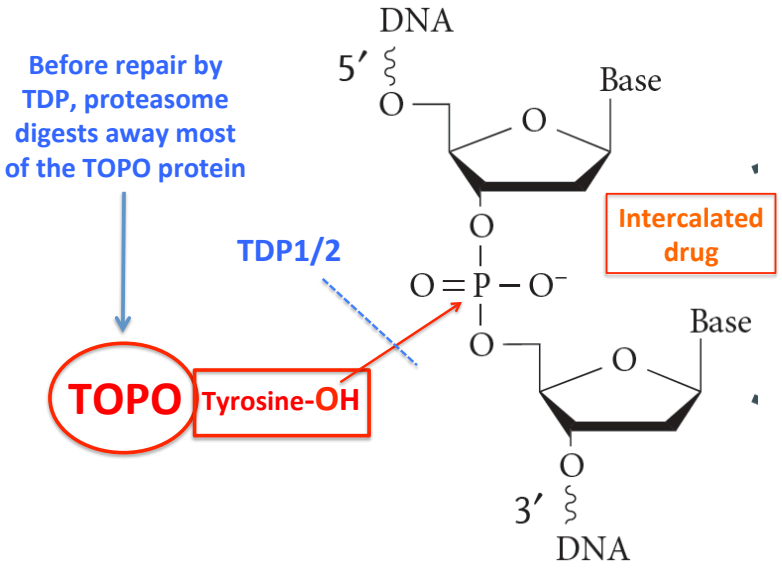

Figure 10.10. How a trapped topoisomerase-DNA complex is repaired by the TDP1 and TDP2 enzymes. The first two steps listed below are part of the normal function of a topoisomerase. The subsequent steps describe what happens if a DNA-intercalating drug traps the topoisomerase on the DNA.
(1) The topoisomerase's tyrosine oxygen atom attacks the phosphorus atom (P) that joins two nucleotide units in a DNA strand (red arrow).
(2) At the same time that the tyrosine oxygen binds to the P, an oxygen atom dissociates from the P, producing a break in the DNA strand. The oxygen atom that dissociates from the P is either the one connected to the 5' part of the DNA or the one connected to the 3' part of the DNA, depending on the type of topoisomerase, but that is a minor point here.
(3) An intercalator-type drug (red box) binds by being plastered against a base-pair adjacent to the strand break and prevents the resealing of the break, thereby trapping the topoisomerase in this never-never state. Some drugs bind to the base towards the 5' part of the DNA strand, and some towards the 3' part of the DNA strand, as shown in Figure 10.6.
(4) A "proteasome" digests away most of the topoisomerase protein.
(5) Finally, TDP1 or TDP2 (depending on the type of topoisomerase) breaks the bond between the tyrosine oxygen atom and the DNA's P atom, while reforming the bond between the P and the previously dissociated DNA oxygen atom. In the end, normal DNA structure has been perfectly restored.

The Etoposide Story

So far, all the Top2-blocking drugs mentioned had the ability to intercalate in DNA, which aided their discovery. But there was a different group of Top2 blockers. Here is the story.

It starts with Hartmann Stahelin and coworkers at Sandoz in Basle, Switzerland, who were manipulating the chemistry of podophyllotoxin, which was known to prevent cells from passing through metaphase of mitosis (Keller-Juslen et al., 1971).

The drug was obtained by extracting it from the roots of a poisonous plant: the American mandrake or Mayapple (Figure 10.11). Podophyllotoxin had anticancer activity in mice but was found to be too toxic for use in patients. Therefore, the chemists at Sandoz made chemical modifications of the compound in search of a less toxic drug. They made almost 50 variations of the chemical structure of podophyllotoxin, several of which increased the survival of mice bearing leukemia L1210.

There was a big surprise, however -- a modest structural change in the podophylotoxin structure completely changed what the drug did in the cell: the toxicity to cell was retained, but the mechanism responsible was entirely different: blocking mitosis was not what caused the cell toxicity. Moreover, the structurally altered drugs were much more effective against cancer.

The chemical change was merely to remove a methyl group and to switch the steric configuration of one of the bonds (Figure 10.12). This modest change eliminated (or greatly reduced) the ability of the drug to inhibit cells in metaphase of mitosis. Instead, the cells were prevented from even starting the process toward mitosis. This was reported by Stahelin in 1970, who surmised correctly that the demethylepipodophyllotoxins (the chemical name of the new compounds) killed cells by an entirely new mechanism (Stahelin, 1970). The new compounds were later discovered to block topoisomerase II.

We became accustomed to that unwieldy chemical name and were pleased to let it fade in memory when it was superseded by new names for the drugs: etoposide and teniposide. (You might suppose that the name “etoposide” referred to its action on topoisomerase, but it seems that name was applied before its action on topoisomerase was known!) Thinking back on this story, the remarkable switch in biological target of action produced by simple changes in chemical structure was remarkable and instructive. It challenged the presumption that the drugs with similar chemical structure would necessarily act on the same target.

Figure 10.11. The American mandrake or mayapple, a poisonous plant, whose roots were the source podophyllotoxin, an inhibitor of mitosis. Chemical modifications of the compound yielded the topoisomerase II blockers and anticancer drugs, etoposide and teniposide. (*Photograph from Wikipedia.*)

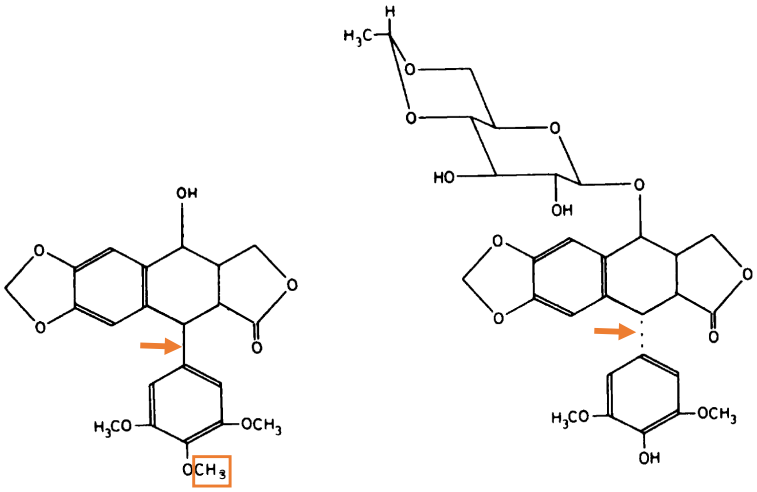

Figure 10.12. Chemical structures of podophyllotoxin and etoposide (VP16). The chemical changes that switched the mode of action were (1) removal of the methyl (CH3) group (red square); and (2) change of the direction of one of the bonds (red arrow), from the bond that points up to the one pointing down relative to the plane of the page. Teniposide (VM26) was a minor chemical modification of etoposide. (The chain in the upper part of the structure on the right was not essential to the change in the manner of the drug's action.)

Thus, the demethylepipodophyllotoxins surprised the researchers, because, although this modest chemical modification of podophyllotoxin increased the ability to extend the survival of mice with cancer, the new compounds did so by an entirely new action. Instead of blocking cells in the middle of mitosis, they instead blocked the ability of cells to begin condensing chromosomes as prelude to entry into mitosis (Grieder et al., 1974). Because of this drastic change in how the new compounds worked, they were given the tentative drug names, VP16 (later, etoposide) and VM26 (later, teniposide).

It was natural to suppose that cells were stopped from starting mitosis by inhibiting DNA synthesis. But the problem with that supposition was that the inhibition of entry into mitosis occurred sooner and at lower drug dose than the inhibition of DNA synthesis (Grieder et al., 1974). Therefore, something other than DNA synthesis inhibition had to be what caused the inhibited cell division. It was a puzzle.

Then, in 1976, Susan Horwitz at Albert Einstein College of Medicine in New York reported that etoposide produced DNA strand breaks that gradually disappeared, presumably by being repaired. But the cause and significance of that finding remained a mystery.

In 1984, Leroy Liu's research group focused their attention on the demethylepipodophyllotoxins – etoposide (VP16) and teniposide (VM26) – because of the chromosome anomalies produced by those drugs. They thought, it seems, that the drugs might be preventing the DNA from untangling at mitosis by inhibiting their newly discovered topoisomerase II (Top2), based in part on our finding that doxorubicin and several other anticancer drugs acted by way of a topoisomerase, in particular, topoisomerase II (Minford et al., 1986; Ross et al., 1979). Sure enough, when they tested the effect of the drug on purified topoisomerase, it was clear that the demethylepipodophyllotoxins inhibited the enzyme (Ross et al., 1984). That was particularly interesting, because, unlike the previously found Top2 inhibitors that were all DNA intercalators (Pommier et al., 1985), the epipodophyllotoxins were thought to lack DNA intercalating activity. The molecules, however, do have polycyclic aromatic groups that may intercalate in the DNA-topoisomerase complex by stacking against a base-pair (Figure 10.12).

In our studies of Top2 inhibitors, we had looked to see what base-pair preference the drug may have for where it traps Top2 in a state where the DNA strands are cleaved. When we tested etoposide and teniposide, we found they had unique preferences for where they trapped Top2 (Figure 10.7) (Pommier et al., 1991).

The mechanism of the reaction seemed to involve an initial interaction between drug and enzyme, rather than between drug and DNA (Burden et al., 1996). Therefore, these Top2-blocking drugs were inferred to act in a manner distinct from the direct DNA-damaging drugs that have Top2 as their target .

Etoposide became one of the most important anticancer drug and was often used in combination with cisplatin or cyclophosphamide; it was found to be particularly effective against small cell lung cancer and testicular cancer (Belani et al., 1994) (Meresse et al., 2004).

The TDP story revisited: cutting off the fuzz at topoisomerase-DNA break sites.

As already explained, topoisomerase-blocking drugs impede the resealing of the normally transient DNA strand breaks that form during normal topoisomerase function. The trouble is that the topoisomerase protein remains persistently bound to the DNA, where its presence blocks repair machinery from coming to the rescue. The topoisomerase cannot dislodge from the DNA in the normal fashion, because the drug, bound to the same site, prevents it from doing so.

The blocked topoisomerase becomes troublesome protein material stuck to the DNA. Protein-digesting machinery was found to come into play to cut away much of the bound topoisomerase molecule but leave behind a DNA-bound protein fragment that it cannot access. The remaining fragment of topoisomerase protein is finally cut away by TDP1 or TDP2 (tyrosyl-DNA-phosphodiesterase 1 and 2), as explained in the legend of Figure 10.10.

The enzymes that came to be known as TDP1 and TDP2 were first discovered in 1996 in yeast by Howard Nash and his coworkers at NIH (Yang et al., 1996) (Pouliot et al., 1999). The process, as conceived by Howard Nash and his colleagues is diagrammed in Figure 10.13 and explained in the Figure's legend as understood at the time.

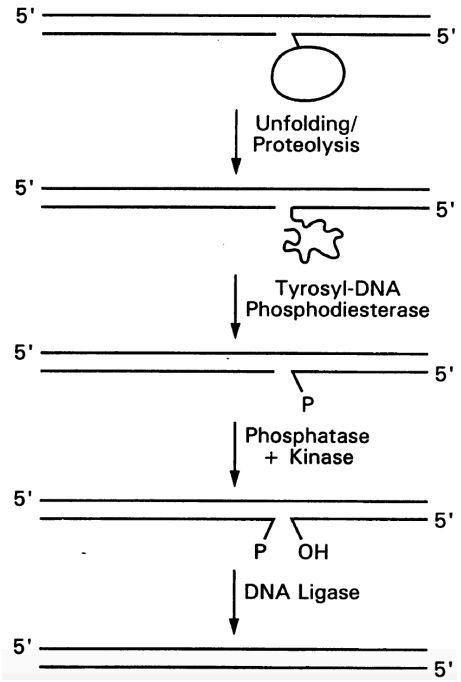

Figure 10.13. The essentials of the process by which a DNA-trapped topoisomerase is removed and the DNA repaired, as surmised by Howard Nash in 1996. The repair is accomplished by tyrosyl-DNA-phosphodiesterase enzymes (TDP1 or TDP2) that Nash discovered. These enzymes removed the topoisomerase fragment from the DNA, so as to allow the DNA strand break to reseal or become repaired (Yang et al., 1996). Shown at the top is a topoisomerase firmly bound to an end of a DNA strand break. A protein-digestion process then removed much of the topoisomerase protein but left behind a DNA-bound fragment that the protease could not reach. TDP1 or TDP2 then would come in in to finish the job of topoisomerase removal.

So, what relevance would the TDP enzymes have for cancer therapy? On further study of the enzyme in yeast, Nash and his coworkers already in 1999 suspected that inhibition of TDP might increase the effectiveness of topoisomerase-inhibiting anti-cancer drugs, because TDP would then not be available to cut away from the DNA break the potentially lethal protein fragment; persistence of the protein link to the DNA could kill the cell -- which would be good if it were a cancer cell that was killed (Pouliot et al., 1999). Therefore, much work was begun to discover TDP-inhibiting drugs that could be tried in cancer therapy together with topoisomerase inhibitors (Pommier et al., 2014).

TDP1 was found to process trapped topoisomerase I, and TDP2 was found to process trapped topoisomerase II (Pommier et al., 2014). The cell, therefore, was normally able to repair both types of topoisomerases trapped by drugs targeted to each of them. Hence, there were therapeutic possibilities for combining a TDP1 or TDP2 inhibitory drug with a drug targeted against the respective topoisomerase.

However, as usual, there were complication. TDP1 could remove trapped topoisomerase I (topic of next chapter) in a camptothecin-treated cell, only if the trapped complex had not yet been encountered by a moving DNA replication machine. If a collision had already occurred, TDP1 was powerless to repair the mess, and a different, more complicated and more imperfect repair process was needed to fix the problem.

It turned out that, in addition to cleaning off trapped topoisomerase complexes from the DNA, the TDP enzymes were able to clean off a variety of other anticancer drugs and toxin molecules that bound and became trapped at the end of a DNA strand break (Pommier et al., 2014).

Summary and further comments.

The discovery that topoisomerases were important targets of anticancer drug action came at a time when those DNA processing enzymes were scarcely known to exist outside of microorganisms. It was one of the few important discoveries to which I could lay claim in nearly 60 years of research on anticancer drug mechanisms. Looking back, I think it a rather remarkable story of a series of unanticipated experiment results that were in no way aimed to the final result.

It all began with my unanticipated discovery – related in the previous chapter -- that large DNA strands from cells dissolved on a filter eluted in a strand size-dependent manner when an alkaline solution was pumped slowly through the filter. By means of quantitative experiments, I was able to use that phenomenon to devise methods to measure the frequencies of DNA strand breaks and DNA-protein crosslinks in drug-treated cells.

The next unanticipated result was that a drug, doxorubicin, that was known to produce DNA strand breaks failed to show any sign of strand breaks in our standard alkaline elution procedure. I thought the failure might be due to doxorubicin producing an excess frequency of DNA-protein crosslinks and that the protein would stick to the filter and prevent the broken DNA strands from eluting. That possibility was confirmed, because digesting away the proteins in the cell lysate beautifully revealed the expected elution of DNA strands.

A puzzle remained however: measurements showed that the drug did not produce enough DNA-protein crosslinks to hide all of the DNA strand breaks produced. Moreover, incredibly, the frequency of DNA-protein crosslinks was equal (within experimental error) to the frequency of the DNA-strand breaks. That seemingly incredible equivalence was also true for two other DNA-intercalating drugs.

Further quantitative considerations led me to conclude that the DNA-protein crosslinks were probably located at the sites of the breaks and, furthermore, that

the DNA-linked protein might in fact be an enzyme that caused the break. That was the first evidence that certain anticancer drugs trap a topoisomerase on the DNA in a state where a DNA strand break exists.

Consequent to that published finding, tremendous interest arose in finding out how various anticancer drugs trap topoisomerases on the DNA and in studying the consequent biological actions, eventually showing that those drug actions on topoisomerases were responsible for the toxic effect on cancer cells for the effectiveness of the drugs in cancer treatment. Those studies were at first of topoisomerase II. The topoisomerase I story is related in the next chapter.

I then wanted to see whether topoisomerase-targeted drugs had individual preference as to where in a DNA nucleotide sequence they most frequently become trapped and cause strand breaks. We found that each drug had its own preference for topoisomerase-trapping, depending on the base-pair at one end or the other of the break. I surmised that the drugs staked against a base-pair at the end of the DNA break, which was consistent with the drugs' capabilities of intercalation in the DNA helix. Each drug had its own preference for the type of base pair on one side or the other to which it preferred to stack against. It was gratifying that our proposed model of drug-trapped topoisomerase II was eventually confirmed by crystallography.

Repair of a persistent DNA strand break that has a drug-trapped topoisomerase bound to the break must first remove the topoisomerase protein from the DNA. Most of the protein was found to be digested away by a proteasome. But a remaining undigested protein fragment remained impervious to removal by proteasome. The enzymes TDP1 and TDP2 then come in to play to complete the removal. The scope of their DNA cleaning abilities was later shown to be much broader in terms of the kinds of strand-break-linked chemical entities they could cut away. Therapeutic applications were contemplated where TDP inhibitors might enhance the potency of drugs that trap DNA at strand breaks created by those drugs.

Much of my work on topoisomerases was carried out together with my colleague and friend, Yves Pommier (Figure 10.14), who, after my retirement as Chief of the Laboratory, went on to carry the studies further as Chief of a newly established Developmental Therapeutics Program at NCI.

Figure 10.14. My colleague and friend, Yves Pommier, had a major role in our topoisomerase studies from a few years after he joined my laboratory as a Research Fellow in 1981 up to the present time as he continues, as Chief of the Developmental Therapeutics Branch of the National Cancer Institute, to investigate many aspects of those remarkable enzymes. Before coming to NCI, Yves had received MD and PhD degrees from the University of Paris.

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